

Citation for published version:

Wang, CZ, Chen, JL, Tang, Y, Zang, Y, Chen, GR, James, TD, Li, J, Wu, C & He, XP 2017, 'Supramolecular polymer dot ensemble for ratiometric detection of lectins and targeted delivery of imaging agents', *ACS Applied Materials and Interfaces*, vol. 9, no. 4, pp. 3272-3276. <https://doi.org/10.1021/acsami.6b14249>

DOI:

[10.1021/acsami.6b14249](https://doi.org/10.1021/acsami.6b14249)

Publication date:

2017

Document Version

Peer reviewed version

[Link to publication](https://doi.org/10.1021/acsami.6b14249)

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Supramolecular Polymer Dot Ensemble for Ratiometric Detection of Lectins and Targeted Delivery of Imaging Agents

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ABSTRACT: A supramolecular, polymer dot based ensemble has been developed for the ratiometric detection of lectins and targeted delivery of glycoprobes. Self-assembly between a blue-emitting polymer dot and a red-emitting glycoprobe, results in an ensemble that shows red emission upon excitation of the polymer dot because of Förster Resonance Energy Transfer. Resulting in ratiometric detection of lectins in buffer solution as well as targeted delivery of the glycoprobe to cells that highly express a sugar receptor. Unlike conventional systems where both the agent and vector are co-delivered intracellularly, our ensemble developed here shows a receptor-controlled dissociation on the cell membrane.

KEYWORDS: Polymer dot, Glycoprobe, Cell imaging, Ratiometric, Probe

Receptor proteins are transmembrane proteins that modulate a number of cellular recognition events through the selective interaction with ligand molecules. In addition to their role in activating cellular pathways, receptor proteins can endocytose specific ligands into the intracellular milieu.¹ The asialoglycoprotein receptor (ASGPr) that is highly expressed on hepatocyte is responsible for the clearance of asialoglycoproteins in the blood. However, ASGPr has been identified as an invasion site for hepatotropic viruses and is overly expressed in liver inflammation.² Therefore, receptor proteins can be exploited as a promising target for imaging-guided disease diagnosis and therapy.

Polymer dots have attracted a great deal of interest due to their excellent optical properties and biocompatibility.³⁻¹⁴ The polymer dots are typically formed by collapsing and folding of hydrophobic polymer chains, and the hydrophobic matrix formed provides an ideal scaffold on which to load hydrophobic compounds. This improves the efficacy of poorly water-soluble compounds. The strong fluorescence of the semiconductor polymer enables researchers to optically track and investigate drug transport and pharmacological effect in real time.

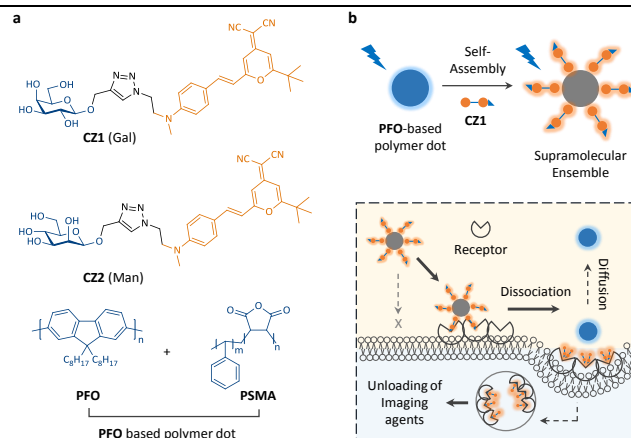


Figure 1. (a) Structures of fluorescent glycoligands (CZ1 and CZ2) and polymer dot (which is formed by mixing poly(9,9-dioctylfluorenyl-2,7-diyl) (PFO) and poly(styrene-co-maleic anhydride) (PSMA)). (b) Schematic illustration of the self-assembly of the supramolecular polymer system and the receptor-controlled dissociation and unloading of an imaging agent.

Here we develop a supramolecular polymer dot based ensemble for the ratiometric detection of lectins and receptor-targeting delivery of imaging agents. The self-assembly between a blue-emitting polymer dot and a red-emitting glycoprobe (sugar-decorated fluorescent probe) produces the ensemble with a red emission upon excitation of the polymer dot (Fig. 1). The ensemble has proven suitable for the ratiometric (emission color from

red to blue) detection of lectins as well as targeted delivery of the glycoprobe to a hepatoma cell line that highly expresses ASGPr. Interestingly, the ensemble shows an ASGPr-mediated dissociation on the cell membrane, leading to the internalization of the imaging agents and the diffusion of the polymer dot vector to the extracellular medium, as determined by stimulated emission depletion super-resolution microscopy (Fig. 1).

The polymer dot based on poly(9,9-dioctylfluorenyl-2,7-diyl) (PFO) (Fig. 1a) was prepared by a reprecipitation method, by mixing PFO with poly(styrene-co-maleic anhydride) (PSMA), as previously described.¹⁵ The addition of PSMA (20% w/w) helps improve the water solubility and stabilize the PFO-based polymer dots formed. The polymer dots are negatively charged and colloidal stable, allowing long-term storage. A red-emitting dicyanomethylene-4H-pyran (DCM) was used to label galactose and mannose, producing the glycoprobes CZ1 (Gal) and CZ2 (Man),¹⁶ respectively, for assembly with PFO. The assembly was achieved by mixing the polymer dots with the glycoligands (CZ1 or CZ2) in pure water, forming the supramolecular CZ/PFO polymer system. We believe that hydrophobic interactions between the DCM moiety of CZs and the aromatic core of PFO-based polymer dots are the main driving force by which the supramolecular ensembles are formed. The PFO polymer is in a folded state, thus leaving hydrophobic cavities to accommodate aromatic compounds. This is supported by previous reports on the formation of hydrophobic-drug-PFO ensembles.¹⁷ Likewise, due to the amphiphilic feature of CZs, the DCM moiety can dock into the hydrophobic cavities of PFO based polymer dots while the hydrophilic sugar parts are exposed to the aqueous solution.

A variety of techniques were used to characterise the supramolecular polymer system. A fluorescence titration assay indicated that the fluorescence of PFO gradually decreased in the presence of increasing CZ1 (Fig. 2a) and CZ2 (Fig. 2d). This suggests that the attachment of CZ to the surface of PFO dots can result in Förster Resonance Energy Transfer (FRET) upon excitation of the latter. This was corroborated by the overlapping absorbance band of CZ and emission band of PFO (Fig. S1). This observation is in agreement with similar reports on the use of polymer dots as FRET donors.¹⁸

To test their biospecificity, we used lectins (proteins that recognise glycoligands) to interact with the supramolecular polymer system. We observed concentration-dependent fluorescence increase and decrease of PFO and CZ, respectively, in the presence of a selective lectin for the supramolecular polymer system (*i.e.* galactose-selective peanut agglutinin (PNA) and mannose-selective concanavalin A (Con A) for CZ1/PFO (Fig. 2b) and CZ2/PFO (Fig. 2e), respectively). This suggests the detachment of CZ from the surface of PFO as a result of lectin recognition, compromising the FRET

mechanism. In addition, the ratiometric fluorescence change (intensity ratio of I_{PFO}/I_{CZ}) was determined to be specific for selective lectins over a range of unselective proteins (Fig. 2c and 2f). This evidence suggests that the supramolecular polymer-dot based systems could dissociate selectively upon interaction with a selective glycoligand receptor.

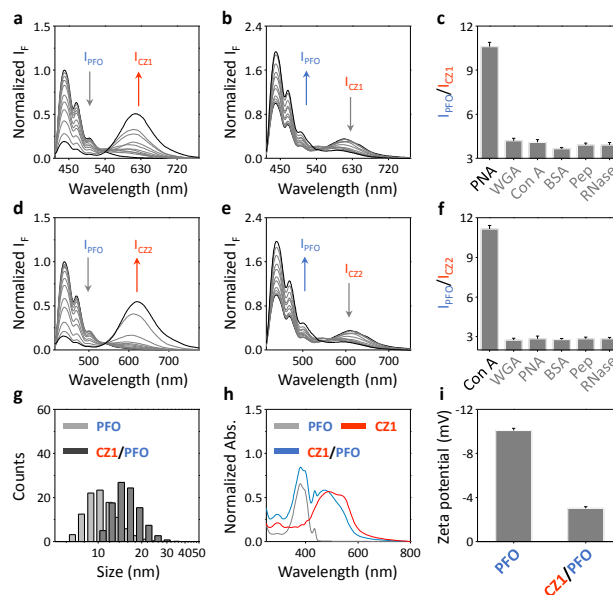


Figure 2. Fluorescence titration of PFO (0.125 ppm) with increasing (a) CZ1 (from the top I_{PFO} curve: 0, 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μ M) and (d) CZ2 (from the top I_{PFO} curve: 0, 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μ M). Fluorescence titration of (b) CZ1/PFO ensemble (1 μ M/0.125 ppm) with increasing peanut agglutinin (PNA; from the bottom I_{PFO} curve: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μ M) and (e) CZ2/PFO ensemble (1 μ M/0.125 ppm) with increasing concanavalin A (Con A; from the bottom I_{PFO} curve: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μ M). Ratiometric fluorescence change of (c) CZ1/PFO ensemble (1 μ M/0.125 ppm) and (f) CZ2/PFO ensemble (1 μ M/0.125 ppm) with different proteins (WGA = wheat germ agglutinin; BSA = bovine serum albumin; Pep = pepsin; RNase = ribonuclease) (the original spectra are shown in Fig. S2). All fluorescence measurements were carried out in Tris-HCl (0.01 M, pH 7.4) with an excitation wavelength of 365 nm. (g) Dynamic light scattering of PFO (0.125 ppm) and the CZ1/PFO ensemble (1 μ M/0.125 ppm). (h) Stacked UV-vis spectra of PFO (50 ppm), CZ1 (100 μ M) and CZ1/PFO ensemble (100 μ M/100 ppm). (i) Zeta potential of PFO (0.125 ppm) and CZ1/PFO ensemble (1 μ M/0.125 ppm). Structures of fluorescent glycoligands (CZ1 and CZ2) and the polymer dot (PFO).

Other techniques including dynamic light scattering (DLS), UV spectroscopy, zeta potential analysis and transmission electron microscopy (TEM) were used to characterise the supramolecular polymer system. DLS indicated that the size distribution of PFO dots slightly increased after CZ1 attachment (Fig. 2g). While the blue-shifted absorption band of the dye (with respect to that of CZ1 alone) suggests the assembly of CZ1 on the PFO

surface (Fig. 2h),¹⁹ the zeta potential of the negatively charged polymer increased after assembly with **CZ1** (Fig. 2i). TEM indicated that assembly with the **CZs** did not change the morphology of the **PFO** nanoparticles (Fig. S3), and confocal microscopy indicated that the blue **PFO** and red **CZ1** emissions were colocalised well on identical dots and clusters (Fig. S4).

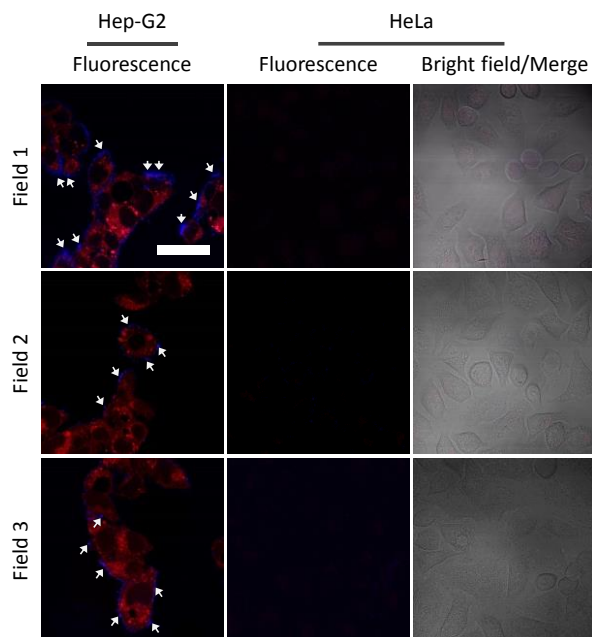


Figure 3. Confocal laser scanning microscopic imaging of Hep-G2 (human hepatoma) and HeLa (human cervical) cells with the **CZ1/PFO** ensemble (14.82 μM /20 ppm) 405 nm (emission: 425-475 nm) and 559 nm (emission: 590-690 nm) for **PFO** and **CZ1**, respectively (Scale bar: 40 μm ; Bright field/Merge means fluorescence merged with bright field; arrows indicate some membrane-adhered **PFO** dots).

With the supramolecular polymer system in hand, we set out to examine its cell imaging ability. The **CZ1/PFO** ensemble was used since the galactoligand of **CZ1** can be recognised by ASGPr. A human hepatoma cell line, Hep-G2, that expresses ASGPr and a human cervical cell line (HeLa) without ASGPr expression were used.^{17,20} Fig. 3 shows the imaging results for **CZ1/PFO** with the two cell lines obtained by confocal laser scanning microscopy. We observed that the red fluorescence of **CZ1** was produced in Hep-G2 rather than in HeLa. Interestingly, the blue-emitting **PFO** dots were observed to be localised on the cell membrane surface of Hep-G2 (for more fields, see Fig. S5), whereas no such blue dots were detected on HeLa.

A set of control assays were also carried out. We determined that knockdown of ASGPr in Hep-G2²¹ significantly reduced the imaging effect of the ensemble (Fig. S6a). Meanwhile, pre-incubation of Hep-G2 with increasing free galactose also competitively diminished the fluorescence (Fig. S6b). These control experiments

suggest that the imaging (release of **CZ1** to Hep-G2 cells) is receptor-targeting. Incubation of **PFO** alone with the cells resulted in the unselective adhesion of the polymer to the membrane of both Hep-G2 and HeLa (Fig. S7). This is contrary to our observation that treatment with the **CZ1/PFO** ensemble did not result in an extensive membrane adhesion of **PFO** to HeLa cells (Fig. 3). These results imply that the presence of the **CZ1** targeting agent could reduce the unselective interaction of **PFO** with the control cells.

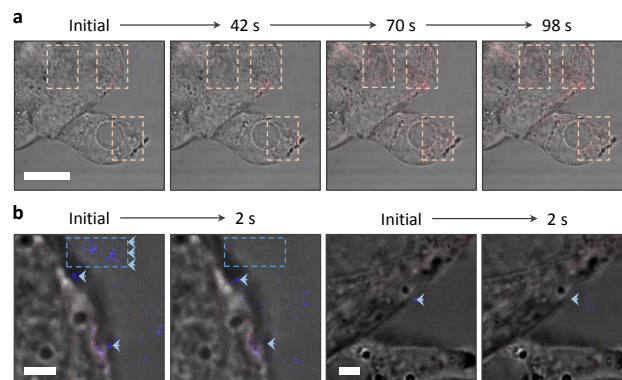


Figure 4. (a) Time-dependent stimulated emission depletion super-resolution microscopy imaging of Hep-G2 (human hepatoma) cells with **CZ1/PFO** ensemble (40 μM /40 ppm) with excitation of 405 nm (emission channel: 418-485 nm) and 552 nm (emission channel: 590-665 nm) for **PFO** and **CZ1**, respectively (Scale bar: 20 μm ; the frames indicate three evident regions that gradually show **CZ1** fluorescence with time; the images that merge fluorescence and bright fields are shown). (b) Enlarged areas of cells to elaborate dynamic localisation of **PFOs** (each set of images contains an initial field and the same field recorded after 2 seconds) (Scale bars: 2 μm).

To evaluate the interaction between the polymer ensemble and Hep-G2, stimulated emission depletion (STED) super-resolution microscopy capable of producing high-resolution, spatiotemporal cell imaging,²² was employed. With STED we carried out a time-dependent imaging assay of **CZ1/PFO** with Hep-G2 (Fig. 4a). The result indicate that the **CZ1** fluorescence was gradually produced with time in a cluster of Hep-G2 cells (the frames indicate three cellular regions with obvious increase in fluorescence over time). However, the **PFO** fluorescence was hardly observed in these cells. Shown in Fig. 4b are two enlarged fields of the cell images, where an initial field and the same field recorded after 2 seconds are displayed to elaborate the dynamic localisation of the **PFO** dots. We observed rapidly diffusing **PFOs** in the extracellular medium rather than in the cells (see also the supplementary video). For example, the dots in the frame (initial) of the first set of images disappeared after two seconds. We also identified membrane-adhered blue dots (initial), which faded with time (2 s).

These imaging results support our hypothesis that the CZ/PFO ensemble dissociates on the membrane surface in a receptor-controlled manner, leading to 1) endocytosis of the fluorescent CZ ligands and 2) the adhesion and then diffusion of PFO to the extracellular milieu. The inability of PFO to penetrate cell membrane might be attributed to its negatively charged nature that is repelled by the phospholipid bilayer of cells. In addition, a preliminary cell viability assay showed that the ensemble was not toxic to Hep-G2 and a human embryonic kidney cell line (HEK293) with increasing concentrations (Fig. 5).

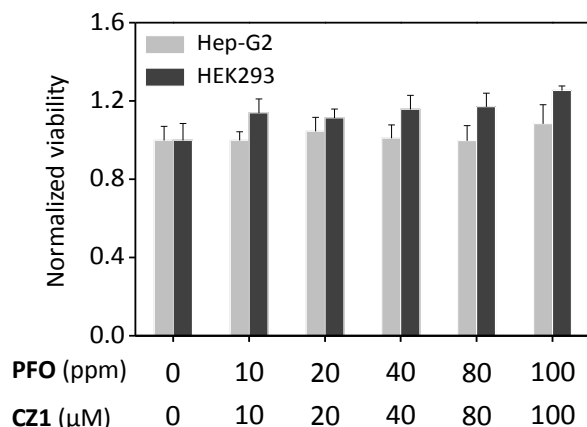


Figure 5. Viability of Hep-G2 (human hepatoma) and HEK293 (human embryonic kidney 293) cells in the presence of increasing PFO and CZ1.

In summary, we have demonstrated the supramolecular assembly of a polymer dot based system for ratiometric detection of lectins and targeted delivery of imaging agents. Besides its ability to detect lectins in a ratiometric fashion, microscopic techniques used suggest that the ensemble could be dissociated on the membrane of a cell that highly expresses a sugar receptor. The dissociation leads to selective, quick endocytosis of the imaging agent and subsequent diffusion of the polymer dot vector to the extracellular milieu. This research has implications for the construction of delivery systems using membrane-impermeable materials as the vector for the targeted delivery of therapeutic agents controlled by receptor-ligand recognition.²³⁻³¹

ASSOCIATED CONTENT

Supporting Information. Additional figures (Fig. S1-S7), experimental section and a video file of STED microscopy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ACKNOWLEDGMENT

This research is supported by the 973 project (2013CB733700), the Science and Technology Commission of Shanghai Municipality (15540723800), the Natural Science Foundation of China (21572058 and 21576088, 61335001 and 61222508) and the Shanghai Rising-Star Program (16QA1401400). The Catalysis And Sensing for our Environment (CASE) network is thanked for research exchange opportunities. T. D. J. thanks ECUST for a guest professorship.

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